Lytic Resistance of Fibrin-Containing Red Blood Cells

Nikolett Wohner, Péter Sótonyi, Raymund Machovich, László Szabó, Kiril Tenkedjiev, Marta M.C.G. Silva, Colin Longstaff, Krasimir Kolev

**Objective**—Arterial thrombi contain variable amounts of red blood cells (RBCs), which interact with fibrinogen through an epifibatide-sensitive receptor and modify the structure of fibrin. In this study, we evaluated the modulator role of RBCs in the lytic susceptibility of fibrin.

**Methods and Results**—If fibrin is formed at increasing RBC counts, scanning electron microscopy evidenced a decrease in fiber diameter from 150 to 96 nm at 40% (v/v) RBCs, an effect susceptible to epifibatide inhibition (restoring 140 nm diameter). RBCs prolonged the lysis time in a homogeneous-phase fibrinolytic assay with tissue plasminogen activator (tPA) by up to 22.7±1.6%, but not in the presence of epifibatide. Confocal laser microscopy using green fluorescent protein–labeled tPA and orange fluorescent fibrin showed that 20% to 40% (v/v) RBCs significantly slowed down the dissolution of the clots. tPA–green fluorescent protein did not accumulate on the surface of fibrin containing RBCs at any cell count above 10%. The presence of RBCs in the clot suppressed the tPA-induced plasminogen activation, resulting in 45% less plasmin generated after 30 minutes of activation at 40% (v/v) RBCs.

**Conclusion**—RBCs confer lytic resistance to fibrin resulting from modified fibrin structure and impaired plasminogen activation through a mechanism that involves epifibatide-sensitive fibrinogen-RBC interactions. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

**Key Words:** blood cells ■ fibrin ■ fibrinolysis ■ plasminogen activators ■ platelet receptor blockers

A reverse correlation between bleeding time and red blood cell (RBC) count was observed more than a century ago.1 When anemic and thrombocytopenic patients were treated with transfusion, the correction of the bleeding time correlated with the corrected hematocrit despite the fall in the platelet count to pretransfusion levels. Later studies confirmed that the prolonged bleeding time of severely anemic patients can be corrected with washed RBC transfusion (thus excluding the effect of plasma factors) at essentially unchanged platelet counts. The mechanism of RBC contribution to the hemostatic function is still open to question, notwithstanding their known role in the maintenance of blood viscosity, the chemical signaling of platelet activation, and the provision of phospholipid support for the activation of coagulation factors (reviewed by Wohner). The solid matrix of hemostatic and pathological blood clots is composed of fibrin and variable cellular elements, but the incorporation of RBCs appears to be mediated through a less specific entrapment than that of platelets.4 Based on binding data, approximately 2% of fibrinogen, the plasma precursor of fibrin monomers, is estimated to circulate in vivo in association with erythrocytes.5 This interaction is not simply a nonspecific protein adhesion to the cell membrane, but it involves an erythrocyte receptor and a specific domain around residues 207 to 303 of fibrinogen Aα-chains. Recently, the RBC receptor for fibrinogen has been identified as an integrin related to the platelet αHβ3 receptor with similar epifibatide sensitivity and impaired function in Glanzmann thrombasthenia. When fibrinogen is converted to fibrin, the presence of RBCs modifies the structural and viscoelastic characteristics of plasma clots.9,10 Because fibrin structure profoundly affects the subsequent removal of blood clots from the vasculature by tissue plasminogen activator (tPA)—dependent proteolysis as a basic fibrinolytic mechanism (reviewed recently by Weisel and Litvinov11 and Lord12), the presence of RBCs may change the lytic susceptibility of thrombi, but no direct evidence for such modulation of fibrin dissolution is currently available. The present study was undertaken in an attempt to characterize the impact of RBCs on 2 distinct stages of the fibrinolytic process, plasminogen activation by tPA and fibrin degradation by the generated plasmid, and correlate it to the observed structural changes caused by the incorporation of RBCs in pure fibrin clots.

**Methods**

**Isolation and Labeling of RBCs**

RBCs were isolated from citrated whole blood collected from healthy volunteers with venipuncture. Within 1 hour after collection,
platelet-rich plasma was removed following centrifugation at 150 g for 10 minutes, and the cell pellet was further centrifuged at 2000 g for 10 minutes to sediment the RBCs. The RBC pellet was washed 3 times with 10 volumes of 1.5 mmol/L KH2PO4, 8.1 mmol/L Na2HPO4 buffer, pH 7.4, containing 137 mmol/L NaCl, 2.7 mmol/L KCl, and 5 mmol/L glucose (PBS) by resuspension and centrifugation at 1200 g for 5 minutes. RBCs were counted using an Abacus Junior B hematology analyzer (Diastron GmbH, Vienna, Austria), and the hematocrit of the RBC suspension was adjusted to 0.8 with PBS. RBCs were stored for up to 2 hours at room temperature before use. For the measurements using confocal laser microscopy, washed RBCs were labeled with Vybrant DiD cell-labeling solution (Invitrogen Life Technologies, Budapest, Hungary) by mixing 5 mL of RBC suspension diluted to a hematocrit of 0.016 in PBS with 25 µL of Vybrant DiD solution for 30 minutes followed by 3 wash cycles (resuspension/centrifugation at 1200 g for 5 minutes) with PBS. On the basis of the cell count, the hematocrit of the Vybrant-labeled RBCs in the final pellet was adjusted to 0.8.

**Ball Sedimentation Assay of Fibrin Dissolution**

The assay was designed as an alternative to the reference method from the European Pharmacopoeia for determination of tPA potency, adapted for the high opacity of clots containing RBCs. Two experimental setups were used, in both of which the total amount of fibrin in the clot was constant, but in Model 1 this was achieved with increasing extracellular concentrations of fibrinogen (from 7.4 to 12.4 µmol/L human fibrinogen, plasminogen depleted, Calbiochem, La Jolla, CA) compensating for the increasing RBC occupancy (from 0 to 40% (v/v)) in a total volume of 2 mL, whereas in Model 2, the extracellular concentration of fibrinogen was constant (7.4 µmol/L) in a clot volume increasing from 0.8 mL in the absence of RBCs up to 1.35 mL at 40% (v/v) RBCs. The RBC-fibrinogen mixtures were supplemented with varying amounts of plasminogen isolated from human plasma, CaCl2, and in certain cases eptifibatide (GlaxoSmithKline Kft, Budapest, Hungary), so that the final extracellular concentrations in the assay clots were always 0.1 µmol/L plasminogen, 1 mmol/L CaCl2, and 20 µmol/L eptifibatide. For all assays, thrombin of low specific activity (Serva, Heidelberg, Germany) was further purified by ion-exchange chromatography on sulfopropyl-Sepharose, yielding a preparation with a specific activity of 2100 IU/mg, and 1 IU/mL was considered equivalent to approximately 10.7 µmol/L by active site titration. Clotting and fibrinolysis were initiated simultaneously in transparent reaction tubes with a diameter of 0.8 cm by mixing RBC-fibrinogen suspensions with PBS containing thrombin and tPA (Boehringer Ingelheim, Ingelheim, Germany) at concentrations needed to reach final extracellular values of 85 and 1 nmol/L, respectively. After 15 seconds, a steel ball with a diameter of 2 mm and a weight of 0.13 g was placed on the surface of the clot, and the time elapsed until the ball reached the bottom of the tube (lysis time) was measured as an indicator of the collapse of identical quantities of fibrin.

**Plasminogen Activation Assays**

For the plasminogen activation assay on the surface of the clot, RBC suspensions in fibrinogen were prepared as described above for the fibrinolytic assay (only plasminogen was applied at a different concentration, so that the final extracellular value in the clots was always 0.5 µmol/L). The RBC-fibrinogen mixtures were clotted with 16 nmol/L thrombin in a total volume of 75 µL in 96-well microplates for 30 minutes. Thereafter, 150 µL of 15 mmol/L tPA was added to the surface of the clots, and after 30 minutes the fluid phase was removed in ice-cold tubes and centrifuged at 2000 g for 10 minutes at 4°C to remove cell debris. The plasmin activity in the supernatant was determined by mixing 100 µL of supernatant and 100 µL of 0.2 mmol/L Spectrozyme-PL (H-0-norleucyl-hexahydrotrorysyl-lysine-p-nitroanilide, American Diagnostica, Pfungstadt, Germany) and measuring absorbance at 405 nm (activity was expressed in ΔA405/min). To evaluate the effect of eptifibatide on plasminogen activation in the absence of fibrin, a 2-stage activation assay was used, as previously described. Briefly, 3 µmol/L plasminogen containing no eptifibatide or 20 µmol/L eptifibatide was mixed with 70 nmol/L tPA, samples were taken at intervals, and the amidolytic activity of the generated plasmin was measured using 0.1 mmol/L Spectrozyme-PL.

**Expression of Fluorescent Chimeric tPA Variant (tPA-GFP)**

Recombinant human tPA-jellyfish green fluorescent protein (GFP) was constructed and expressed using the Bac-to-Bac baculovirus expression system as a tPA-C-terminal fusion with enhanced GFP isolated from the pEGFP plasmid (Clontech, Mountain View, CA), as described previously.

**Confocal Laser Scanning Imaging in the Course of Fibrinolysis**

RBC suspensions in fibrinogen were prepared as described for the plasminogen activation assay using Vybrant-labeled RBCs and replacing 1% of the fibrinogen with Alexa Fluor 546–conjugated fibrinogen (Invitrogen Life Technologies, Budapest, Hungary). Fibrinogen was clotted with 16 nmol/L thrombin for 30 minutes in 0.5-mm-high chambers constructed from glass slides. Thereafter, 60 nmol/L IPA-GFP was added to the edge of the clot, and the fluorescence (excitation wavelength 488 nm, emission wavelength 525 nm for tPA-GFP detection; excitation wavelength 543 nm, emission wavelength 575 nm for Alexa Fluor 546–fibrinogen detection; excitation wavelength 633 nm, emission wavelength 650 nm for Vybrant-labeled RBC detection) was monitored with Confocal Laser Scanning System LSM510 (Carl Zeiss GmbH, Jena, Germany) using 510 (tPA) or 546 (fibrinogen) nm for Vybrant-labeled RBC detection; monitored with Confocal Laser Scanning System LSM510 (Carl Zeiss GmbH, Jena, Germany) using 510 (tPA) or 546 (fibrinogen) nm for Vybrant-labeled RBC detection.

**Scanning Electron Microscope Imaging of Thrombi and Fibrin**

The RBC-fibrinogen mixtures were clotted with 16 nmol/L thrombin in a total volume of 100 µL, and after 30 minutes clots were placed into 10 mL of 100 mmol/L Na-cacodylate, pH 7.2, buffer for 24 hours at 4°C. Following repeated washes with the same buffer, samples were fixed in 1% (v/v) glutaraldehyde for 16 hours. The fixed samples were dehydrated in a series of ethanol dilutions (20 to 96% (v/v)) in a T1 mixture of 96% (v/v) ethanol/acetone, and pure acetone, followed by critical point drying with CO2 in a E3000 Critical Point Drying Apparatus (Quorum Technologies, Newhaven, United Kingdom). The specimens were mounted on adhesive carbon discs and sputter coated with gold using a SC7620 Sputter Coater (Quorum Technologies), and images were taken with an EVO40 scanning electron microscope (Carl Zeiss GmbH).

**Morphometric Analysis of Fibrin Structure and Statistical Procedures**

The scanning electron microscope images of thrombi and fibrin were analyzed to determine the diameter of the fibrin fibers using self-designed scripts running under the Image Processing Toolbox, version 7.0, of Matlab 7.10.0.499 (R2010a) (Mathworks, Natick, MA). For the diameter measurements, a grid was drawn over the image with 10 to 15 equally spaced horizontal lines, and all fibers crossed by them were included in the analysis. The diameters were measured by manually placing the pointer of the Distance tool over the end points of transverse cross-sections of 300 fibers from each image (always perpendicularly to the longitudinal axis of the fibers). The distribution of the data on fiber diameter was analyzed using an algorithm described previously for identification of a theoretical distribution that gives the best global fit to several empirical data sets. The best-fitted distributions for different samples were compared using the Kuiper test, and probability values were calculated with Monte Carlo simulation procedures. When a statistically significant difference between 2 distributions was established, the numeric characteristics of the central tendency and variance were considered to be statistically significant. The statistical hypothesis
testing for differences in other experimental measurements in this report was performed with the Kolmogorov-Smirnov test (Statistical Toolbox 7.3 of Matlab).

**Results**

Thrombi formed in vivo under similar rheological conditions in large arteries showed remarkable differences in their RBC content (Supplemental Figure I, available online at http://atvb.ahajournals.org). Even within a single thrombus, regions of high and low RBC count could be identified indicating the prevalence of local factors (blood vessel geometry, flow pattern) as determinants of thrombus composition. Thus, a correct understanding of the impact of RBCs on the lytic susceptibility of such compartmental architecture should be based on data modeling the structure and lysis of fibrin over a range of relevant RBC counts. The present study was restricted to clots formed from purified fibrinogen and washed RBCs. Because of the known compartmentalization of fibrin and cellular components within thrombi, extreme model states were evaluated (Figure 1). At increasing RBC occupancy, identical amounts of fibrin were present either in clots with constant total volume (and consequently increasing extracellular fibrin concentration; Model 1) or in clots with volume expanding proportionally to the increase of RBC content (preserving constant extracellular fibrin concentration; Model 2). In all cases, the intercellular fibrin was composed of thinner fibers and smaller pores, resulting in a denser network compared with the cell-free fibrin, as illustrated for Model 1 in Figure 2 (Supplemental Figure II).

The role of the integrin-dependent RBC-fibrinogen interaction in the modification of fibrin structure was approached with the addition of the integrin blocker eptifibatide (Figure 2A) at a concentration that has been shown to be efficient in the inhibition of fibrinogen binding to RBCs. Because the most striking changes in the fibrin structure related to the presence of RBCs and eptifibatide appeared to be in the fiber diameter, this parameter was quantitatively evaluated using the morphometric approach illustrated in Figure 2B. In all cases, the increase in the clot occupancy by RBCs was coupled to a decrease in the fiber diameter (Table). This effect was more pronounced in Model 1 (at increasing extracellular fibrinogen concentrations), where 40% (v/v) RBC occupancy resulted in a more than 2-fold diameter increase compared to the cell-free fibrin. The role of the integrin-dependent RBC-fibrinogen interaction in the modification of fibrin structure was approached with the addition of the integrin blocker eptifibatide (Figure 2A) at a concentration that has been shown to be efficient in the inhibition of fibrinogen binding to RBCs. Because the most striking changes in the fibrin structure related to the presence of RBCs and eptifibatide appeared to be in the fiber diameter, this parameter was quantitatively evaluated using the morphometric approach illustrated in Figure 2B. In all cases, the increase in the clot occupancy by RBCs was coupled to a decrease in the fiber diameter (Table). This effect was more pronounced in Model 1 (at increasing extracellular fibrinogen concentrations), where 40% (v/v) RBC occupancy resulted in a more than 2-fold diameter increase compared to the cell-free fibrin.
When fibrinogen containing plasminogen is mixed with thrombin and tPA, following clotting the plasminogen activator is uniformly dispersed in the total volume of the clot and the generated plasmin dissolves the fibrin matrix. As a result of proteolysis, fibrin loses its mechanical stability, and thus a steel ball placed on the top of the clot will descend in response to gravity. This global lytic assay system is intimately related to the biological function of fibrin, because it reflects the changes in the mechanical stability of the fibrin matrix. The time to achieve complete collapse of the clot can be used as a global end-point indicator of this assay. If identical quantities of fibrin are degraded, this lysis time depends on the combined effect of several factors (stability of the initial fibrin network, rate of plasminogen activation, catalytic efficiency of plasmin in various fibrin structures). Although the constant amount of fibrin was maintained differently in our Models 1 and 2 defined above, in both situations the presence of 10% and 40% (v/v) RBCs slowed down the fibrinolysis induced by incorporated tPA, and eptifibatide partially or completely reversed the RBC effect (Figure 3). In view of our recent data on the differential impact of fibrin structure on plasminogen activation and fibrin dissolution, the discordant response of the 20% (v/v) RBC-fibrin clots in this complex fibrinolytic assay indicated the necessity to dissect the role of RBCs in these 2 stages of the process.

When tPA was applied to the surface of fibrin containing plasminogen and increasing numbers of RBCs, plasmin was generated on a fibrin surface with decreasing area (part of the interface was occupied by RBCs) and varying structure (Figure 2 and the Table), which is also known to affect plasminogen activation. Under the conditions of Model 1, after a 30-minute activation on the surface of fibrin containing 10% to 20% (v/v) RBCs, the plasmin level peaked at 36.6% to 44.6% over the cell-free control values, but further increase in the RBC content moderated this stimulation to 25.6% at 40% (v/v) RBCs (Figure 4A). It is noteworthy that on the surface of cell-free fibrin formed from fibrinogen at concentrations equivalent to the extracellular values in Model 1, plasmin generation decreased with increasing fibrinogen concentrations (at 12.4 μmol/L fibrinogen, 22.2% less plasmin formed than at 7.4 μmol/L fibrinogen; data not shown). In contrast to the bell-shaped dependence of plasminogen activation on the RBC count in Model 1, a linear decrease in the amount of plasmin generated could be observed at increasing RBC occupancy and constant extracellular fibrinogen concentration (Figure 4B). Because the RBC-occupied area of the activation interface was the same in both models for identical volume occupancy, the differences in plasmin generation can be attributed to the variations in fibrin structure. In line with this, eptifibatide at 20 μmol/L moderately enhanced the effect of the highest RBC counts in both models of fibrin-dependent plasminogen activation (in the absence of fibrin, eptifibatide modestly stimulated the tPA-induced plasminogen activation, resulting in 20% more plasmin at 30 minutes; data not shown).

The interplay of tPA, fibrin, and RBCs at the fluid phase–clot interface was approached with confocal microscopic observation of tPA-GFP penetration in the clot, propagation of the lytic front of orange fluorescent–labeled fibrin, and release of fluorescent-labeled RBCs from the clot. In agreement of our and others’ earlier findings, within 10 minutes after...
application of tPA-GFP to the surface of preformed fibrin, a sharp zone of intense tPA-related fluorescence was formed on the surface of cell-free fibrin (Figure 5A) and preserved throughout the lysis observation period (Figure 5B). In contrast, the presence of RBCs prevented this accumulation of tPA (Figure 5A and 5B). The progress of fibrin dissolution could be monitored on the basis of the position of the boundary layer of the fluorescent fibrin phase (Figure 5C). The presence of RBCs at 20% to 40% in the clot significantly slowed down the dissolution of fibrin after a 25-minute exposure to tPA, with the greatest difference at 30 minutes (3.5-fold shorter distance run by the lytic front), followed by a catch-up phase, but the difference remaining almost 2-fold at 55 minutes. Eptifibatide did not affect the progress of lysis in the absence of RBCs, but it moderated the inhibiting effect of RBCs. The release of RBCs from the clot occurred in parallel with the progress of the lytic process, as evidenced by the lack of RBCs in the fluid phase in the early stage of dissolution (Figure 5A) and their abundance out of the clot at later stages (Figure 5B).

Discussion

The hemostatic function of whole blood is a delicate balance of coagulation and fibrinolytic pathways, which is profoundly...
affected by RBCs. Although severely anemic patients show a bleeding trend, which can be reversed with transfusion of washed RBCs, the increased RBC mass of polycythemia vera patients is coupled to frequent (in more than one third of the patients) thrombotic complications. However, the local number of RBCs in thrombi does not necessarily correlate with the systemic RBC count, because some complicating factors, such as flow conditions and variable geometry, especially at stenotic sites, will influence the distribution of the cellular elements in the lumen of blood vessels. Thus, not surprisingly, variable amounts of RBCs can be detected in thrombi removed surgically from large arteries (Supplemental Figure I). The major goal of the present study was to evaluate the contribution of RBCs to clot stability from the aspect of fibrinolysis. Because in a plasma environment RBCs could affect the final clot structure through interactions at multiple steps of the coagulation process (eg, exposing negatively charged phospholipids that provide surface for the assembly of coagulation factor complexes) and to avoid the interference of plasma inhibitors, we evaluated clots prepared from purified fibrinogen and washed RBCs. Thus, initiating dissolution with plasminogen and tPA at precisely known concentrations a relatively limited number of variables needed to be considered in the evaluation of the fibrinolytic side of the hemostatic balance. The study of clot dissolution was approached in 2 experimental setups. In the model with incorporated tPA, the activator was uniformly dispersed within the RBC-fibrin clots at a concentration of 1 nmol/L, which is relevant to the amount of tPA measured in human arterial thrombi, thus mimicking the situation in hemostatic plugs at sites of vascular injury when coagulation and fibrinolysis are initiated simultaneously. In the second setup, tPA was applied to the surface of preformed RBC-fibrin clots at a concentration of 60 nmol/L, relevant to the values maintained in blood in the course of enzymatic thrombolysis, thus mimicking the therapeutic systemic administration of plasminogen activators. Despite the differences in the experimental approaches, in both settings the effect of RBCs was consistent; increasing RBC occupancy of the clot resulted in resistance to lysis (Figures 3 and 5C). This antifibrinolytic effect of RBCs, which is expressed at physiologically relevant RBC counts, shifts the balance of coagulation/fibrinolysis in favor of clotting and may prevent the premature dissolution of hemostatic plugs or contribute to the thrombotic complications in polycythemia vera. In addition, the heterogeneous RBC composition of thrombi (Supplemental Figure I) may be one of the factors contributing to the variability in the lytic susceptibility during thrombolytic therapy of myocardial infarction and stroke. The mechanism of RBC-related fibrinolytic resistance was approach using ultrastructural and kinetic methods. Scanning electron microscope imaging of the fibrin structures formed in the presence of RBCs evidenced thinner fibers in the RBC-rich areas (Figure 2). These measurements were performed in 2 extreme settings: either keeping the total amount of fibrin constant, with a consequent increase in the extracellular fibrinogen concentration in parallel with the RBC-excluded volume, or at a constant extracellular fibrinogen concentration for any RBC occupancy (Figure 1). In the second setting, a dose-dependent decrease in fiber diameter was observed with increasing RBC occupancy of the clot (Table), whereas in the first setting this effect was counteracted by the thickening effect of rising fibrinogen concentration (Table, reference values), with the outcome seen in Figure 2B and the Table (Model 1, +RBC values). The lysis time measured in the ball sedimentation assay (Figure 3) was inversely correlated with the established changes in fiber diameter and not directly with the RBC occupancy of the clots. When epifibatide, a blocker of the RBC fibrinogen receptor, reversed the RBC effect on the fiber structure (Figure 2 and the Table), it also reversed the RBC-related inhibition of fibrinolysis (Figures 3 and 5C). Thus, RBC-induced modification of fibrin structure appears to be a major
mechanism in the lytic resistance of clots with RBC content. These results are in agreement with earlier findings that if the fiber diameter is reduced by different factors, the overall rate of fibrin dissolution is slower in similar lytic models with clot-incorporated or surface-applied activators.

Because the lytic assays discussed above reflect a global measure of the combined outcome of the 2 stages of fibrinolysis (plasminogen activation and proteolysis of fibrin), separate kinetic and confocal microscopic measurements were performed in attempt to identify discrete effects of RBCs. The plasmin generated in the course of plasminogen activation on the surface of clots in Model 1 increased in the presence of RBCs in the clot (Figure 4A), which is surprising in view of the RBC-related suppression of fibrin dissolution. Furthermore, plasminogen activation on the surface of clots in Model 2 shows a dose-dependent decreasing trend at rising RBC occupancy (Figure 4B). These discordant findings can be explained by the simultaneous action of 2 opposing factors. First, greater RBC occupancy of clots results in thinner fibers (Table), which are a better template for tPA-catalyzed plasminogen activation. Second, the presence of more RBCs in the clot volume results in more cells on the clot surface and a consequent relative increase in the area devoid of plasminogen and inert in terms of plasmin generation. The reduction in interface area occupied by plasminogen-presenting fibrin translates into less plasmin generation. In the setting of the Figure 4A assay, the thinner fiber network gains higher density at increasing fibrinogen concentrations, and thus, the effect of template quality coupled to higher cofactor concentration dominates. In the setting of a constant fibrinogen concentration (Figure 4B), increasing the surface taken by RBCs limits plasminogen activation despite the thinner fibers. Independently of the variations in plasmin generation, in both setups, RBCs slow down fibrin dissolution (Figure 3), which suggests that the plasmin-catalyzed proteolytic stage prevails in the control of fibrinolysis at increasing RBC occupancy. The confocal images of fibrin exposed to tPA-GFP (Figure 5A and 5B) provide direct evidence for the limited access of tPA to activatable substrate; the accumulation of tPA in the interfacial fibrin layer is definitely retarded in the presence of RBCs.

This interpretation of the observed changes in plasminogen activation on the surface of RBC-fibrin clots is further supported by the effects of epifibatide in these assays (Figure 4). This blocker of the RBC fibrinogen receptor increases the fiber diameter (Table) and thus eliminates RBC-related changes in the structure of the fibrin template that favor plasminogen activation, leaving unopposed the effect of the expanding RBC-occupied surface with a consequent decline in plasminogen activation. In the absence of fibrin, epifibatide stimulates plasminogen activation (data not shown), and this effect persists if activation occurs on the surface of cell-free fibrin with less efficient template function (thicker fibers at 8.2 μmol/L or higher fibrinogen concentration, Figure 4A), but not on the thinner fibers with better cofactor function in the tPA-catalyzed plasminogen activation (Figure 4B). The reported observations for the generation of plasmin in the presence of RBCs indicate that binding of the receptor blocker to RBCs eliminates its direct effect on plasminogen activation and likewise moderates the RBC effects. The data on the effects of epifibatide in plasminogen activation (Figure 4) and fibrinolytic (Figure 3) assays suggest that the RBC fibrinogen receptor mediates, at least in part, the RBC-related lytic resistance of fibrin.

In summary, our present report unravels a clot-stabilizing function for RBCs based on suppressed tPA-induced fibrinolysis in RBC-modified fibrin structures. In addition to steric hindrance, RBCs modulate fibrinolysis through a specific fibrinogen receptor, as evidenced by the effects of epifibatide, a cyclic heptapeptide that reversibly inhibits the binding of fibrinogen to αIIbβ3 integrin. This αIIbβ3 antagonist is used in clinical practice as an antiplatelet agent, but its newly described role in RBC-containing clots extends our understanding of its favorable antithrombotic action by overcoming the resistance to lysis of RBC-rich thrombi.

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Disclosures

None.

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For illustration of the variable RBC content of thrombi two extreme cases (low and high RBC count) were selected out of 33 thrombi removed with surgery from large arteries. Both samples were from graft vessels (JJ, femoro-popliteal by-pass Dacron graft; BOM, ileo-femoral by-pass homograft from deceased healthy donor). All laboratory findings were in the normal range except for the moderate anemia of BOM (0.29 hematocrit, 9.1 g/dL hemoglobin). No inherited or acquired thrombophilic state was diagnosed in the reported patients. At the time of thrombectomy both patients received heparin treatment. Written informed consent was obtained from all patients and the study protocol was approved by the institutional and regional ethical board. Immediately (within 5 min) after the surgery 5x5x10 mm pieces of thrombi were cut, washed, fixed and dehydrated as detailed in Methods and SEM images were taken. The cellular elements are predominantly RBCs.
Fig. II Area of pores in the fibrin network

Pore area of the fibrin clots presented in Fig. 2 is evaluated according to algorithm described earlier. The numbers under the RBC content show the median and interquartile range of the fitted distributions for the pore size. The presence of RBC is coupled to smaller pore size (the difference between 10 and 20, as well as 20 and 40 % RBC is not significant, for the rest of the pairs \( p < 0.05 \)). A limitation of the approach is related to the fact that when RBC are present, the term pore loses its original meaning in the pure fibrin. RBC fills in the pore, so it’s not empty and the evaluation algorithm catches only a fraction of the real pores and not those where RBC occupy the background. Thus, at higher RBC occupancy the distribution is based on a limited number of data, which does not allow reliable estimation of differences between different experimental setups. However, the jump in pore size between cell-free and RBC containing fibrin is large and conclusively supported by the data.
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